Co-dispersion of chia plant protein and casein micelles in milk

Thesis

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Abstract

Food proteins are the basis for imparting functionality in many foods. Not all proteins are able to form associations with other proteins, but the casein micelles present properties that make them a unique system for protein blending. This has the potential to provide novel functionalities in industrial food applications. To capitalize on this, our study investigated processing methods to stabilize the interactions of casein micelles in milk with chia protein. Most plant proteins are not soluble in water, so a co-dispersion of casein micelles with these globular proteins was sought via homogenization. The temperature of the skim milk was lowered, and sodium citrate was added to allow the casein micelles to dissociate enough as to interact with the chia protein. The soluble chia protein was extracted from microfine powder of the seed. The fiber, starch material, and pericarp were precipitated and separated. The supernatant containing the dispersed chia protein was added to the skim milk. This was homogenized under cold condition (4 °C) and relatively higher pressure (350 bar) to increase the stability of the colloidal dispersion by applying shear. The particle size, zeta potential, and total protein were analyzed before and after homogenization. The zeta potential increased significantly after homogenization from a mean value of 33.98 mV to 45.83 mV. This indicates a stable co-dispersion of the chia protein and casein micelles. Overall, dissociating casein micelles and homogenizing at colder temperatures and higher pressures may effectively co-disperse and stabilize chia plant protein and casein micelles for use in a wide range of dairy applications.

This is dedicated to Dwight.

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Chapter 1: Introduction

1.1 Background

Dairy consumption has been declining for decades, and fluid milk prices are expected to continue increasing [14]. Now more than ever, consumers are making more informed decisions about what they eat and drink. They demand dairy companies to express more creativity and innovation in their product portfolios. As such, dairy producers are investing in research and development to develop novel ways to attract consumers [7].

Trends defining the food and beverage sector include instant nutrition, everyday performance foods, health-enhancing food processing, high protein [2], nutrient-dense, clean label, functional foods and beverages [23]. There is an emphasis on functional and naturally processed foods and beverages. Consumers are finding importance in knowing the protein source, reading labels, and demanding more details on products. Currently, the big trend in protein is plant-based proteins. However, dairy proteins still have an advantage because most plant proteins are generally not as nutritionally complete [1]. The global high-protein based food and beverage market is large and can be tapped into with the innovation of a functional, dairy-based product. As such, this study explores the interactions between chia plant protein and casein micelles.

1.2 Protein

Second to water, proteins are the most abundant substance in the body. Because essential amino acids cannot be made by the body, they must be consumed. Sources of complete proteins include milk [1] and chia. This means they provide all the essential amino acids.

Proteins impart functionality in many foods. Examples of these functionalities include emulsification, foaming, gelling, heat stability, viscosity, texture, and fat- and waterbinding [25] [5]. Protein interactions are of interest and of importance in industry because these associations can lead to novel functionalities in industrial food applications [18].

1.2.1 Casein micelles

Casein proteins are well-studied in the field of dairy science [13]. In bovine milk, casein proteins are polydisperse, colloidal particles called "casein micelles". They have properties that make them a unique system for protein blending: they dissociate with the addition of calcium chelating agents, swell at low temperatures [3], and swell at alkaline pH in the range of 6.6 to 8.5 [22]. Adding calcium chelating agents to casein micelle dispersions results in the dissociation of the micelles into smaller casein aggregates by inducing solubilization of colloidal calcium phosphate from the casein micelles [15] [6]. The dissociation of casein micelles changes their technological properties. Examples of chelating agents include sodium phosphate, ethylenediaminetetraacetic acid (EDTA), and sodium citrate.

The properties of casein micelles promise novel applications in the functional food and pharmaceutical industries. An example includes the encapsulation of health-related substances in casein matrices [9].

1.2.2 Chia protein

Chia is a complete source of protein. With 20% protein content, it has twice the amount of protein as any other seed or grain. As such, chia seeds have a huge potential to improve nutrition and reduce malnutrition due to a lack of protein. Globulins are the main proteins in chia seed, which comprises approximately 52% of the total protein content along with mainly 7S and 11S proteins. The proteins in chia, mainly albumins and globulins, have better thermal stability than other proteins. Additionally, chia is gluten-free. This unique feature allows patients with celiac disease to consume chia [26].

1.3 Research Significance

Chia seed is currently experiencing a renaissance [26]. It has twice the amount of protein as any other seed or grain, five times the amount of calcium of milk, twice the amount of potassium as bananas, thrice the reported antioxidant strength of blueberries, and three times more iron than spinach [24]. Chia is a complete source of protein. Additionally, it is high in soluble fiber as well as omega-3 and omega-6 fatty acids. The chia to be used in the experiment is Anutra ^{TM} microfine chia powder, which is non-GMO and approved by the USDA and FDA [10]. With all these added benefits from chia, a high-protein and functional dairy-based beverage could be developed. However, the challenge with most plant proteins is their low solubility in water [27].

Casein micelles present properties that make them a unique system for protein blending. To capitalize on this, our study investigated processing methods to stabilize the interactions of casein micelles in milk with chia protein. Most plant proteins are not soluble in water, so a co-dispersion of casein micelles with these globular proteins was sought via homogenization. Protein-protein interactions are of interest to industry, as these have potential for novel functionalities in food and beverage applications.

Chapter 2: Literature Review

2.1 Casein structure

Casein proteins are the main phosphoprotein in milk. They are comprised of several casein fractions that are distinguished via electrophoresis: α-, β-, and κ-caseins [11]. Casein micelles form as spherical structures, which allows the casein to remain suspended in the milk. The hydrophilic parts of casein molcules form the outside of the casein micelle, and the hydrophobic parts form the core. Calcium and phosphorous are bound inside the micelle. The structure of the casein micelles can be easily disrupted or changed with exposure to heat or changes in acidity [19]. The size, form, and structure of casein micelles are important in the milk industry [13].

There are several proposed models to represent casein micelle structure. This is due in part to the effects of various agents and conditions on the stability of casein micelles [5]. Casein micelles have a spherical shape and a diameter that ranges from 50 to 500 nm, with an average diameter of 120 nm [13]. Every proposed model of casein micelle structure has κ-casein on the surface. Because of the steric hindrance and electrostatic repulsion, there is stability and negative charge [5].

2.2 Protein isolation

2.2.1 Centrifugation

In a study by Wirths, centrifugation was used to isolate both the soluble and insoluble protein fractions [28]. The protein suspension was centrifuged at 17,000 g at 4 °C for 20 min. The supernatant, which is the soluble fraction, was separated from the pellet via decanting and stored at 4 °C. The pellet was resuspended and centrifuged again. Then, the supernatant was separated from the pellet via decanting and stored at 4 °C.

2.2.2 Isoelectric precipitation

The pH of a solution where the net primary charge of a protein is zero is called the isoelectric point. Above this pH, the protein's surface is mostly negatively charged. Thus, similarly-charged molecules exhibit repulsion. Below this pH, the protein's surface is predominantly positively charged, and there is repulsion between proteins. At the isoelectric point, attraction forces predominate and cause proteins to aggregate and precipitate.

Chia seeds have 22 to 24 g/100 g protein, which is a good proportion of protein to extract using the isoelectric method at different pHs. In a study by Cardenas et al., chia protein concentrate was prepared with the following procedure [17]. Defatted cashew kernel flour (DCF) was stirred with deionized water, which was adjusted to a pH of 8.0 with 1 M NaOH, for 2 hours at 25 °C. The flour to water ratio was 1 to 20 (w/v%). This slurry was centrifuged in an Eppendorf centrifuge (USA) at 5000 g for 30 minutes at 25 °C. The pellet with the insoluble chia protein was resuspended in deionized water, which had its pH adjusted as described above. This was centrifuged

again. The supernatants were combined, and the pH was adjusted to 3.0; 4.0; 5.0; and 6.0 (isoelectric point). These samples were stored at 4 °C for 48 hours with 1 M HCl. Then, they were centrifuged at 5000 g at 25 °C for 30 minutes. The precipitate was washed and resolubilized with deionized water that was adjusted to a pH of 7 with 1 M NaOH at 25 °C. Then, this was dialyzed against water and lyophilized. Finally, the lyophilized protein samples were stored in plastic bottles at -20 °C [17].

2.3 Sample preparation

Sodium azide was used for milk preservation during the experiment. In a study by Sinaga et al., casein micelle size after the addition of 0.02-0.10% sodium azide was analyzed. Sodium azide did not affect the properties of milk during storage, including the size of the casein micelles [21]. On the same day and after 14 days in storage, sodium azide did result in any significant changes to the average casein size. 0.02% sodium azide had a narrower size distribution compared with that of the control and the milk samples with other concentrations of sodium azide. The role of sodium azide in this experiment was to prevent microbial growth over the course of the skim milk's ageing.

2.4 Adjusting pH

In a study by Liu et al., results showed the casein micelle structure is more compact at low pH and expanded at high pH. Casein micelles can self-assemble in the pH ranges of 2.0 to 3.0 and 5.5 to 12.0 [16]. In a study by Sinaga et al., casein micelles were reported to swell at alkaline pH in the range of 6.6 to 8.5 [22]. Differences in the casein micelle size were reported to be reversible in the pH range of 6.0 to 7.0.

Properties, including soluble calcium and milk whiteness, were restored. However, it remains uncertain if the reversibility in size was related to the restoration of native casein micelle structure.

Adjustments to the pH of milk affects the internal structure of casein micelles. Above the isoelectric point of the casein protein molecules, the casein micelles are predominately negatively charged. The increased repulsion produces loose and more open structures [9].

2.5 Calcium chelation

2.5.1 Sodium citrate

Sodium citrate, the sodium salt of citric acid, induces solubilization of colloidal calcium phosphate from casein micelles, resulting in the dissociation of casein micelles [20]. In a study by Heertje et al., the addition of sodium citrate resulted in the partial breakdown of the casein micelle while forming smaller particles [12]. There were also large particles present that had looser, expanded structures compared to those of the original casein micelles. This phenomenon can be explained by the release of small particles (most likely β- and κ-casein) from the casein micelles, which leaves behind a micellar framework of α_s- caseins and does not affect the structural integrity of the original particle. Figures 2.1 and 2.2 show the effects of adding 0.02 M sodium citrate.

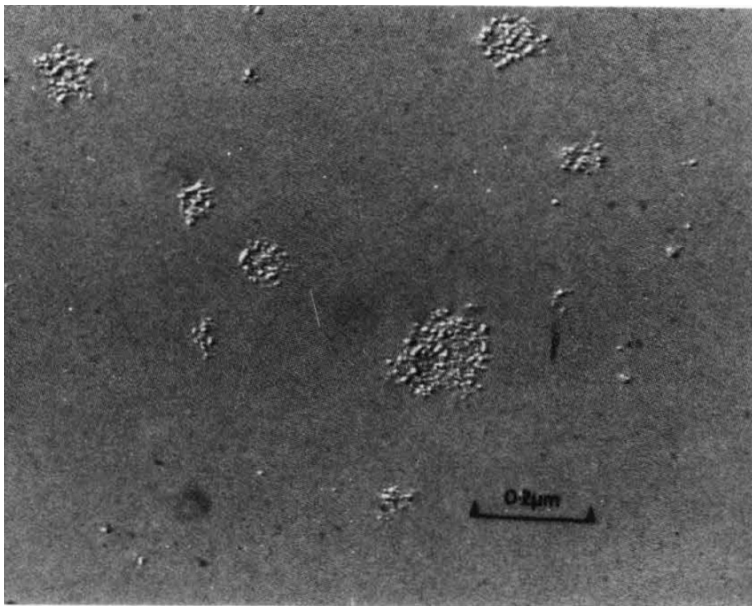

Figure 2.1: Casein micelles in milk with tightly packed structures [12].

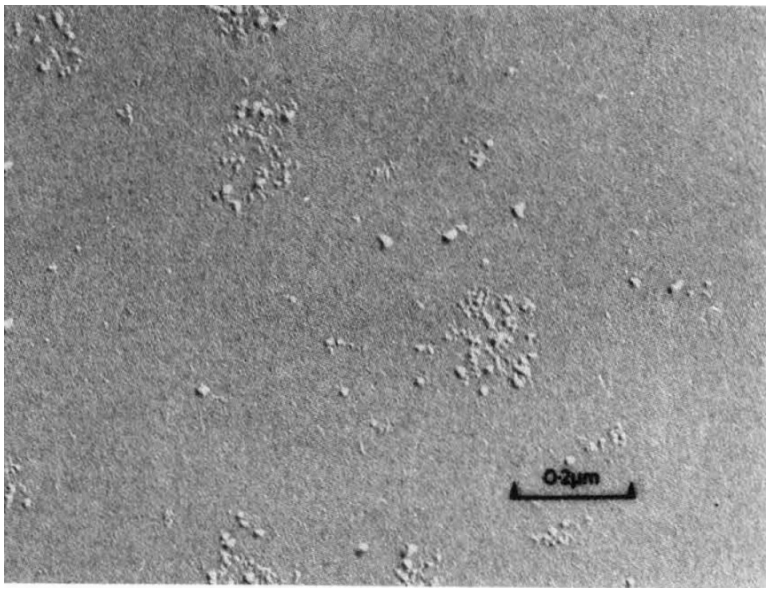

Figure 2.2: Casein micelles in milk treated with 0.02 M sodium citrate, exhibiting looser structures [12].

2.6 Applying shear

For homogenization, we can generate shear forces in various ways. Examples of equipment used for homogenization include:

1. High shear mixers
2. Colloid mills
3. High pressure homogenizers

With high pressure homogenizers, homogenization is achieved by applying shear forces to the fluid due to velocity gradients. The mixture is forced to flow with high velocity through a narrow gap. Other ways to apply shear include mechanical agitation and ultrasonic vibrations [4].

2.7 Dynamic light scattering

Dynamic light scattering (DLS) is used to measure particle size and particle size distribution, typically in the submicron region. DLS can be used for molecules, including proteins, carbohydrates, micelles, polymers, and nanoparticles [13]. This method can also quantify the stability of colloidal suspensions.

Chapter 3: Methodology

3.1 Chia protein extraction

To extract the soluble chia protein from the Anutra ™ microfine chia powder, the chia powder was soaked in reverse osmosis water at 37 °C with agitation at a speed of 70 rpm. After 24 hours, the mixture was transferred to 250 mL polypropylene centrifuge bottles. The bottles were centrifuged at 10,000 rpm for 10 minutes. Then, the supernatant with the soluble chia protein was separated from the pellet via decanting and stored at 4 °C for later use. The pellets were resuspended in reverse osmosis water and agitated at 37 °C and a speed of 70 rpm for 24 hours. The supernatant was separated from the pellet again.

3.2 Sample preparation

All sample solutions were prepared with a target sodium azide and sodium citrate concentration according to the following procedure.

1. Weigh out 518 g skim milk.
2. Add 0.02% sodium azide.
3. Add 0.59 mmol/g sodium citrate.

4. Equilibriate overnight (at least 24 hours).

The supernatant with soluble chia protein was combined with the prepared skim milk sample. This mixture was homogenized at 4 °C and 350 bar.

3.3 Ingredients

The two main ingredients used in the experiments were Kroger Brand skim milk and Anutra ™ Microfine Chia Powder. Sodium citrate from Archer Daniels Midland Company was used to dissociate the casein micelles.

3.4 Equipment

For this study, the following equipment were used.

3.4.1 Potential analyzer

The NanoBrook ZetaPALS potential analyzer was selected to determine the stability of the colloidal suspension of the chia protein and casein micelles in milk. It utilizes phase analysis light scattering and is used for biological samples, including proteins. It was chosen because it is extremely sensitive relative to other techniques, and it was available in the lab. A higher magnitude of the Z-potential indicates a more stable colloid. Results can generally be interpreted as follows:

- 0 to ± 5, Rapid coagulation or flocculation
- ± 10 to ± 30 Incipient instability
- ± 30 to ± 40 Moderate stability
- ± 40 to ± 60 Good stability

- More than ± 61 Excellent stability [8].

This equipment also measured particle size, which was useful to characterize the casein in milk and the proteins in the chia powder.

3.4.2 Protein analyzer

The CEM Sprint Rapid Protein Analyzer was used to measure the protein in the samples. It provides quick results for dairy products and is more repeatable than Kjeldahl and combustion techniques. It does not measure total nitrogen.

3.4.3 Rotovapor

The Buchi R-200 Rotavapor System was used to concentrate the soluble chia protein content in the supernatant. The heated bath was set to 50 ° C. The set up used a 24/40 500 mL evaporating flask, 24/40 250 mL rotary evaporator bump trap, and a vacuum pump.

3.4.4 Homogenizer

A GEA Niro Soavi TwinPanda 400 homogenizer was used to apply shear to the test sample. The operating conditions were 350 bar and 4 °C.

Chapter 4: Results

4.1 Altering casein micelle structure

Changes in the size of the casein micelles were observed over the course of 14 days. The particle diameter of the control skim milk was 203.08 nm. After adding sodium citrate and allowing the solution to equilibriate for 24 hours at 4 °C, the particle diameter increased significantly to 249.09 nm. After five days, the particle diameter was 247.31 nm, which is relatively similar to the particle size after one day and still significantly larger than that of the control. Figure 4.1 shows the significant increase in particle diameter with the addition of sodium citrate and storage at 4 °C.

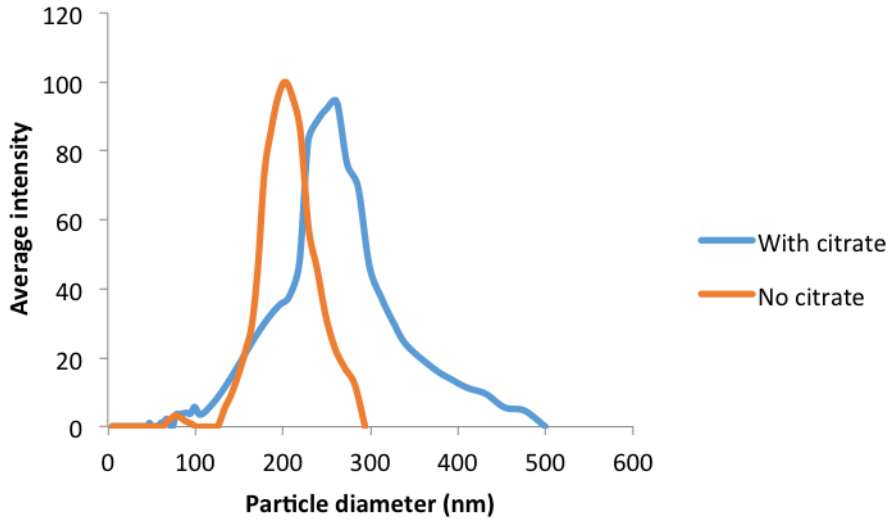

Figure 4.1: Changes in particle size of casein micelles with addition of sodium citrate.

The particle diameters of casein micelles in the control skim milk, soluble chia protein, mixture of the skim milk and supernatant, homogenized sample at 0 days, and homogenized sample after 14 days are shown in Figure 4.2. The control, shown in orange, had two peaks at 131.13 nm and 287.21 nm. The supernatant with the soluble chia protein exhibited two peaks at 796.78 nm and 4,981.96 nm. After combining the skim milk with the soluble chia protein, the particle diameter was 180.02 nm. Homogenization at 4 °C and 350 bar resulted in a particle diameter of 218.99 nm. This peak had a more narrow distribution than prior to homogenization. After 14 days, the particle diameter increased to 236.36 nm but still had a relatively similar distribution.

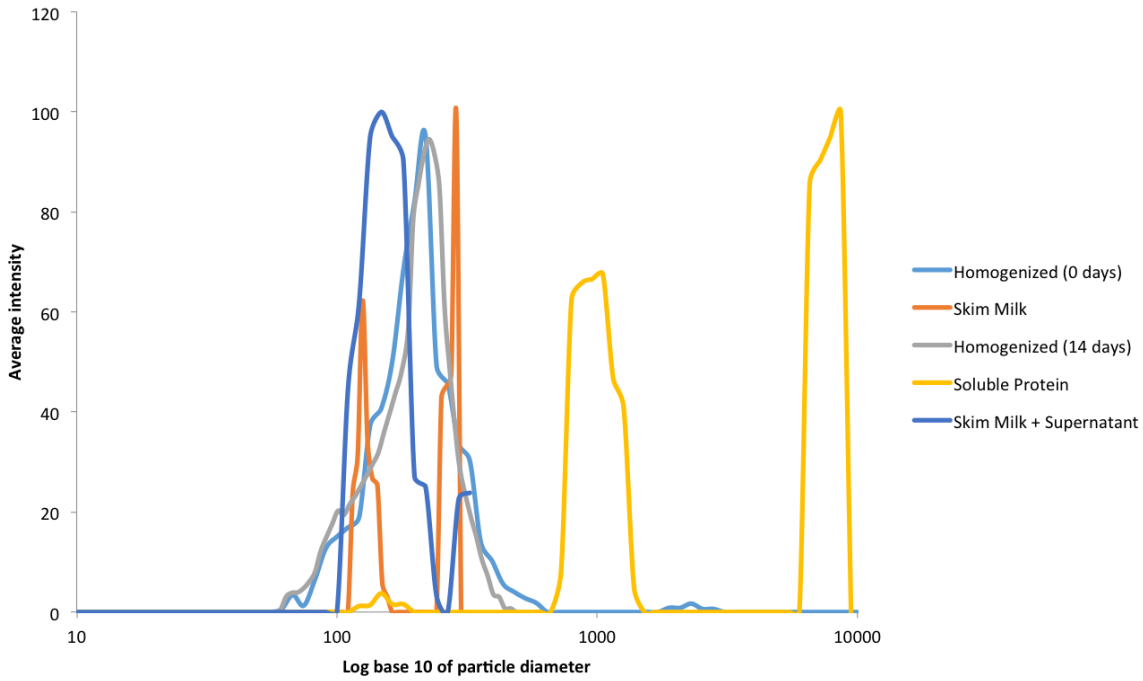

Figure 4.2: Changes in particle size with processing via homogenization over 14 days.

Combining the chia protein and the casein micelles appeared to have some protein-protein interactions. The large size of the particles in the supernatant with soluble chia protein decreased significantly with the addition of skim milk. Applying shear to the mixture of chia protein and casein protein resulted in a more narrow particle size distribution.

4.2 Stabilization of system

The zeta potential was used to quantify the stability of the colloidal dispersions. The stability of the chia protein and casein micelles are summarized in Table 4.1. Skim milk and soluble chia protein had a Z-potential value of 33.98 mV, which indicates moderate stability. After homogenizing at 4 °C and 350 bar, the Z-potential increased

to 45.83 mV. This value is significantly higher than the control, suggesting higher stability. After two weeks, the Z-potential of the homogenized solution was 41.82 mV. This is still significantly more stable than the control.

Table 4.1: Quantifying stability of the mixture over the course of 14 days.

Sample	**Z-potential (mV)**
Skim milk + soluble protein	33.98*
Homogenized (0 days)	45.83*
Homogenized (14 days)	41.82*

Applying shear via homogenizing at 4 °C and 350 bar significantly improved the stability of the chia and casein protein system.

4.3 Statistical Analysis

JMP statistical software was used to analyze the data using a significance level of 0.05. An analysis of variance (ANOVA) test indicated that the addition of sodium citrate and storage at 4 °C significantly increased the measured particle diameter of the casein micelles. Another ANOVA test showed that homogenization significantly improved the stability of the chia and casein protein beverage system.

Chapter 5: Conclusion

Dissociating casein micelles with sodium citrate and storing the sample at 4 °C significantly increased the particle diameter of the casein micelles from 203.08 nm to 249.09 nm. After five days, the particle diameter was 247.31 nm, which is relatively similar to the particle size after one day and still significantly larger than that of the control. Homogenizing the soluble chia protein and milk under cold condition (4 °C) and relatively higher pressure (350 bar) significantly increased the stability of the mixture, resulting in a co-dispersion and stabilization of chia plant protein and casein micelles. The Z-potential significantly increased from 33.98 mV to 45.83 mV after homogenization. After two weeks, the Z-potential was 41.82 mV, which is still significantly more stable than the control.

The results suggest a stable, co-dispersion of the chia plant protein and casein micelles was achieved through the proposed processing method. This study demonstrates the potential use of homogenization at cold temperature and high pressure to enhance the stability of natural plant-based beverages for dairy-based applications.

5.1 Future Work

Due to time constraints, many experiments have been left for the future. This thesis mainly focused on the preliminary testing of natural processing methods on

a chia protein and dairy protein system. It was determined that dissociating and swelling the casein micelles prior to homogenizing the chia protein and dairy protein mixture at cold temperature and high pressure results in a stable beverage system. Results from the experiments led to potential ideas to improve the experimental design. The following ideas can be explored:

1. Isolate and concentrate the protein from the chia powder with isoelectric precipitation.

2. Quantify the protein content in the supernatant and pellet with a nitrogen analyzer.

3. Determine how much protein from the chia powder is recovered in supernatant compared to to that in the pellet after centrifugation.

4. Determine what kind of proteins from chia are in each fraction. Perform an amino acid analysis of both fractions.

5. Measure the particle diameter of casein micelles after restoring calcium and temperature.

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